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(54) **DIFFERENTIATION OF HUMAN EMBRYONIC STEM CELLS**

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(57) **ABSTRACT**

The present invention provides methods to promote the differentiation of pluripotent stem cells into insulin producing cells. In particular, the present invention provides a method to produce cells expressing markers characteristic of the pancreatic endocrine lineage that co-express NKX6.1 and insulin and minimal amounts of glucagon.

14 Claims, 9 Drawing Sheets

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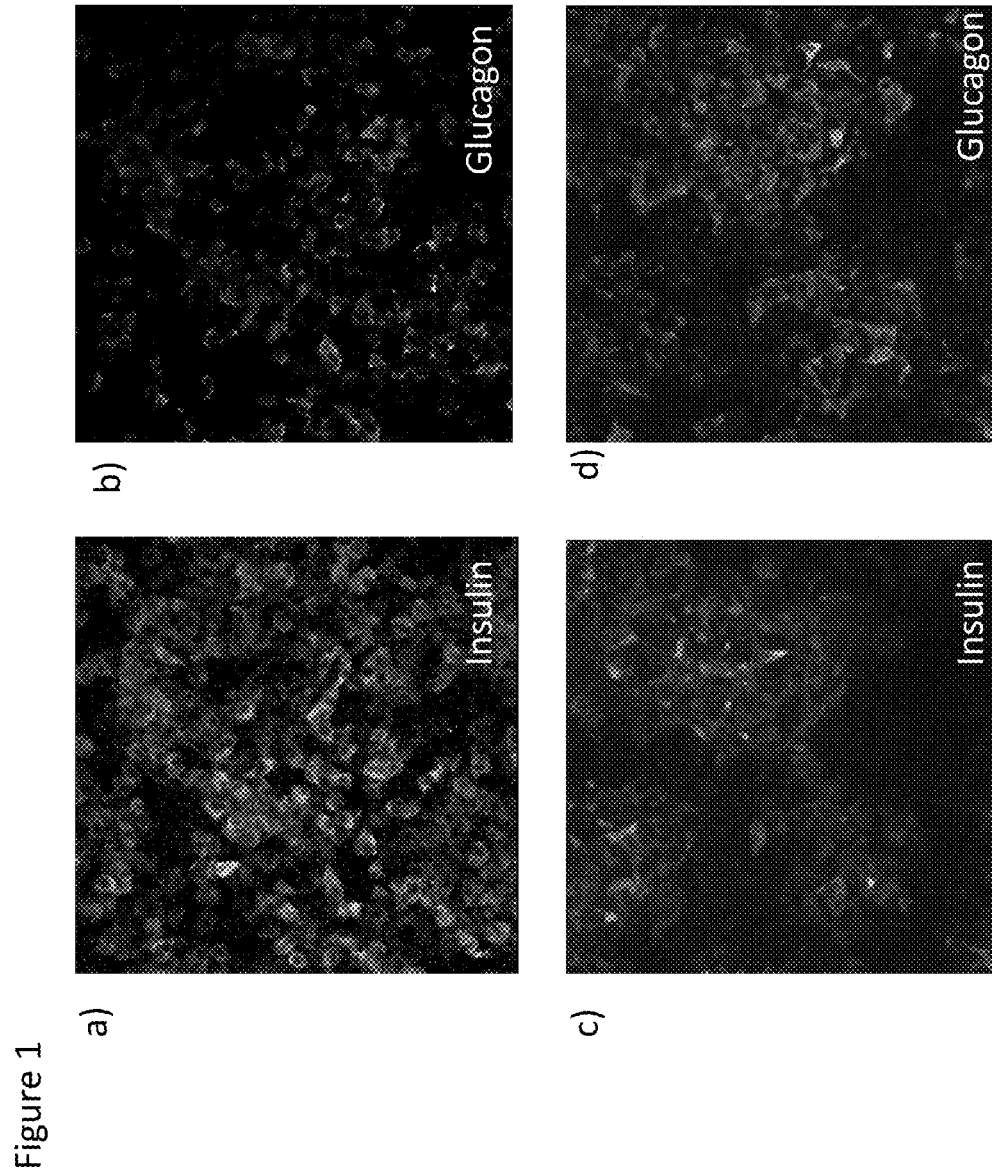
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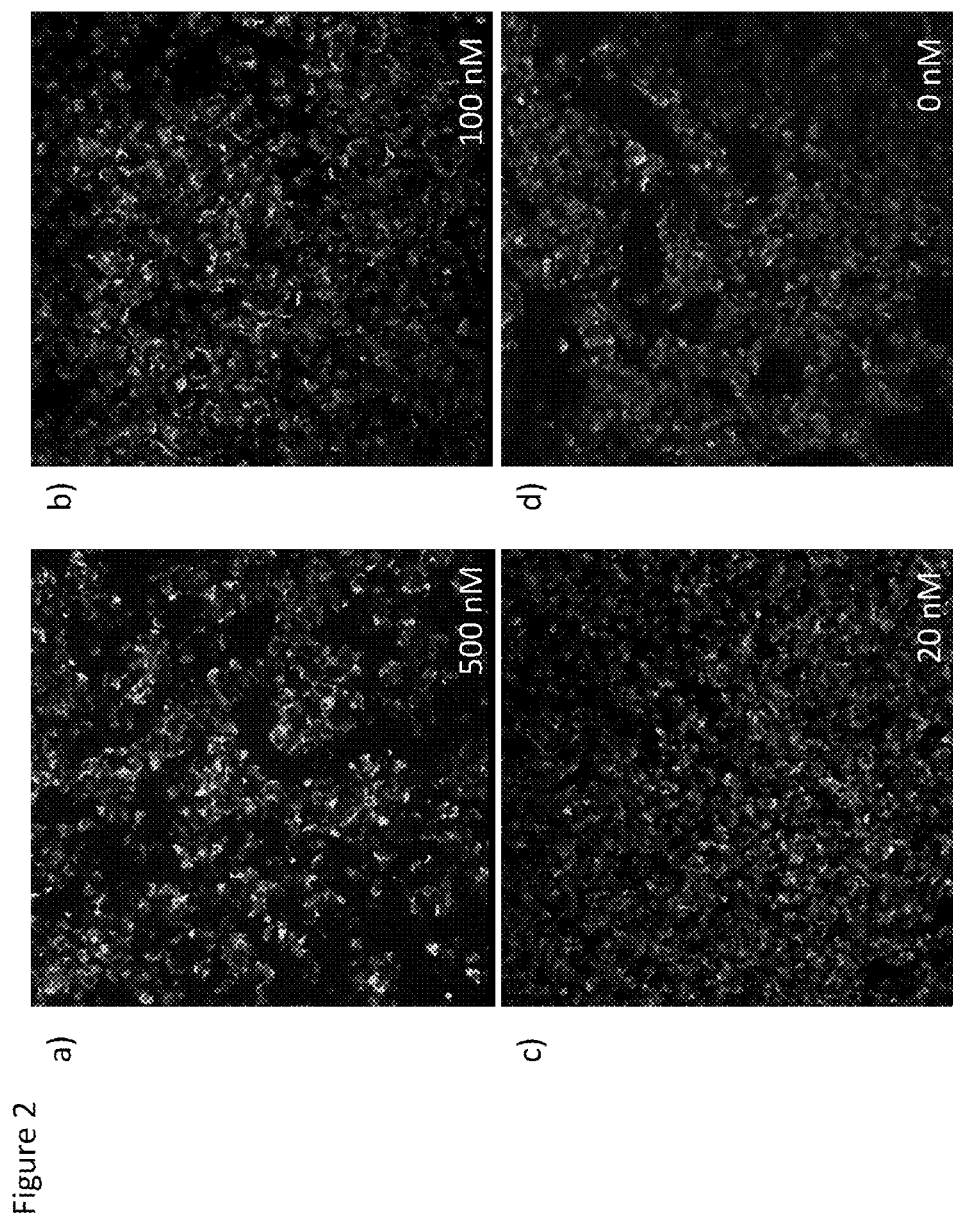
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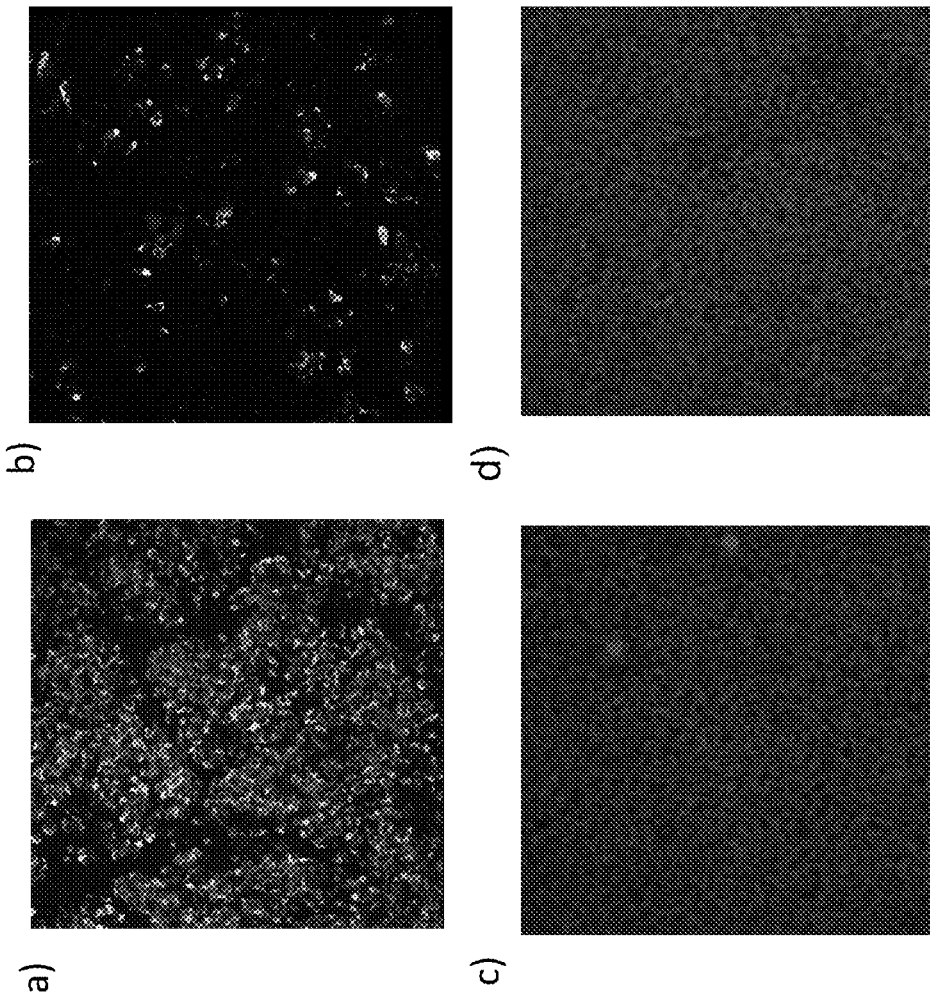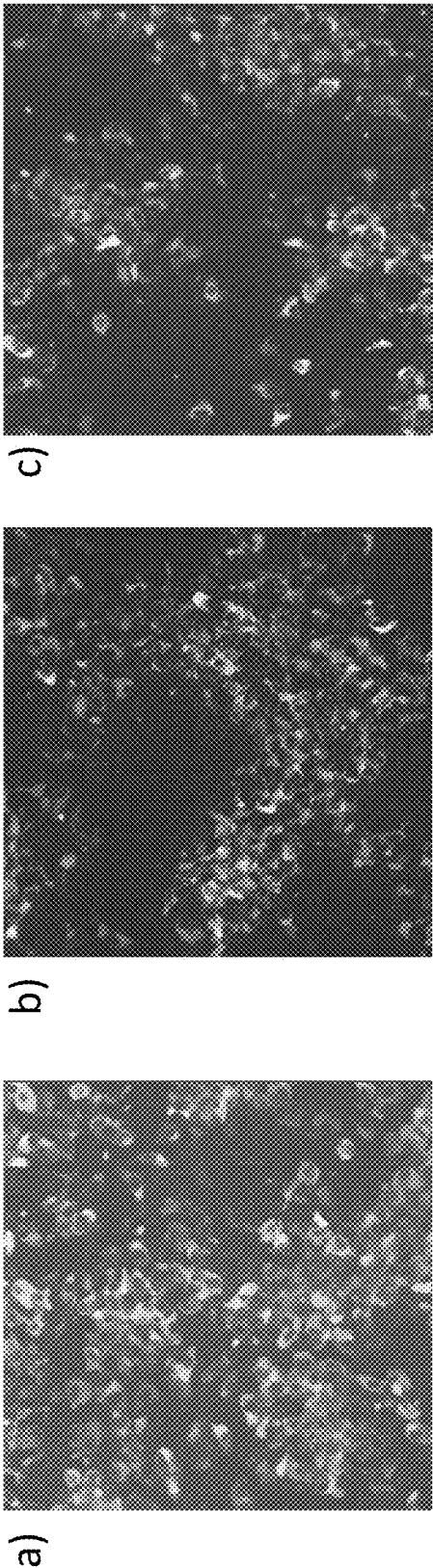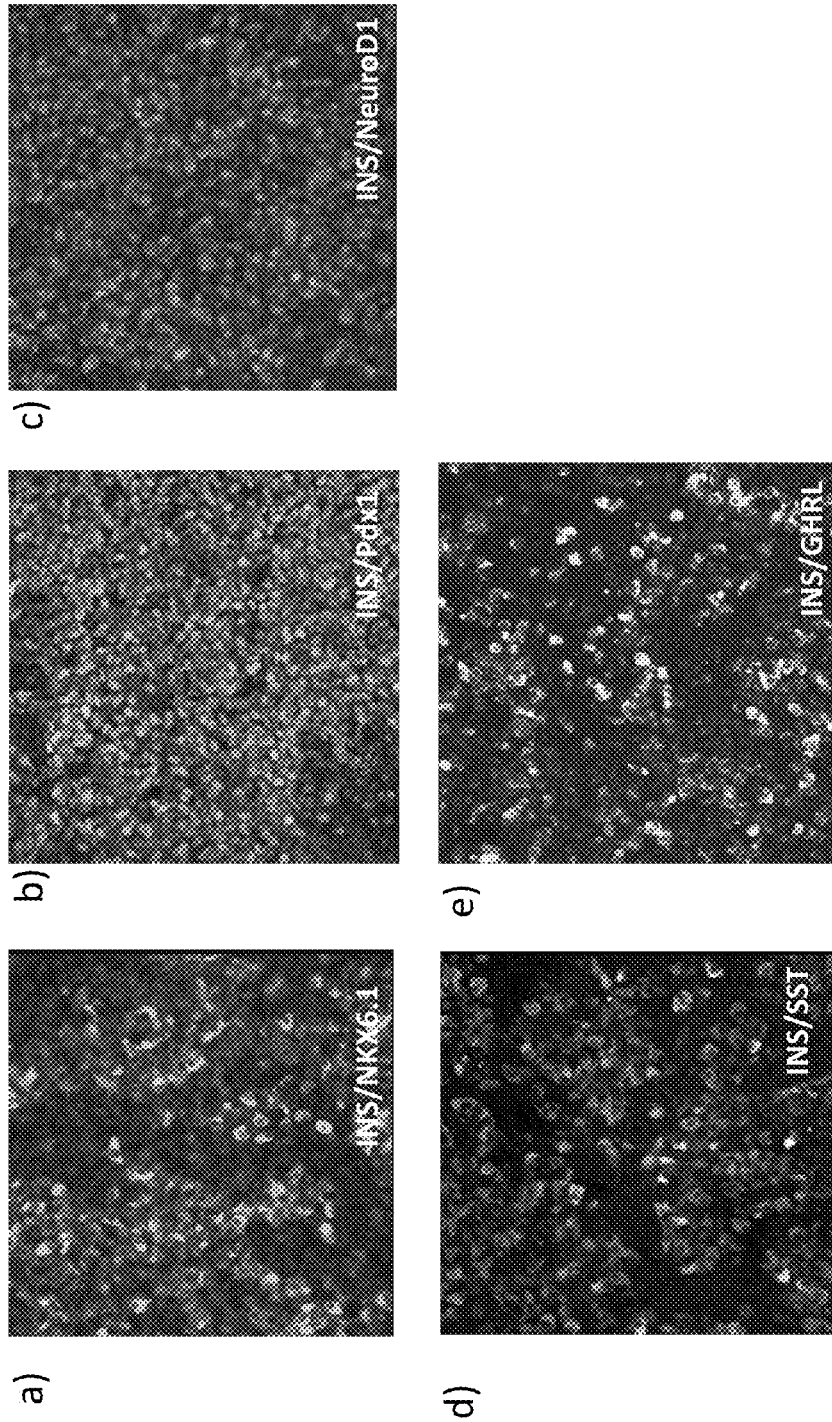
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Figure 1

Figure 2

Figure 3
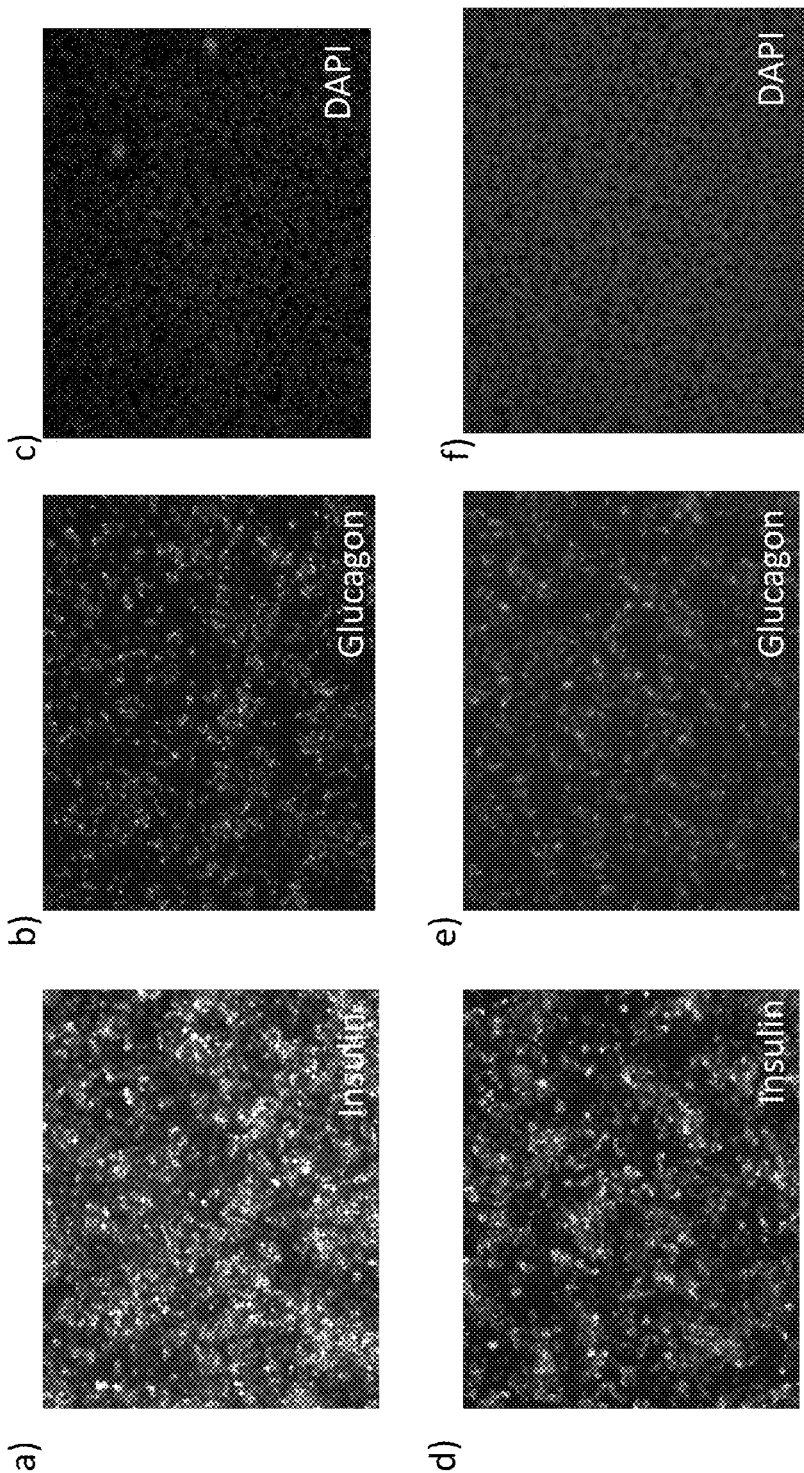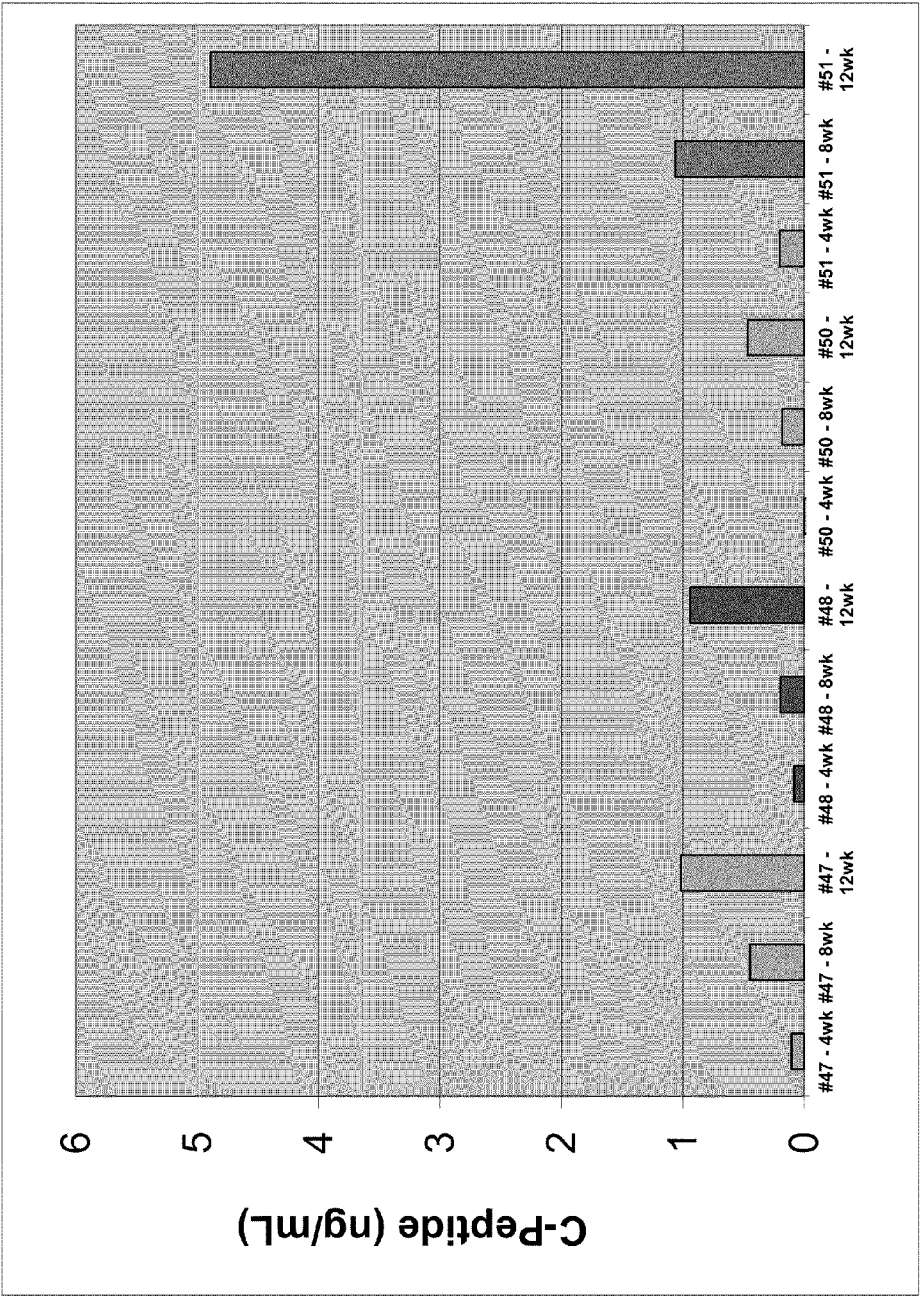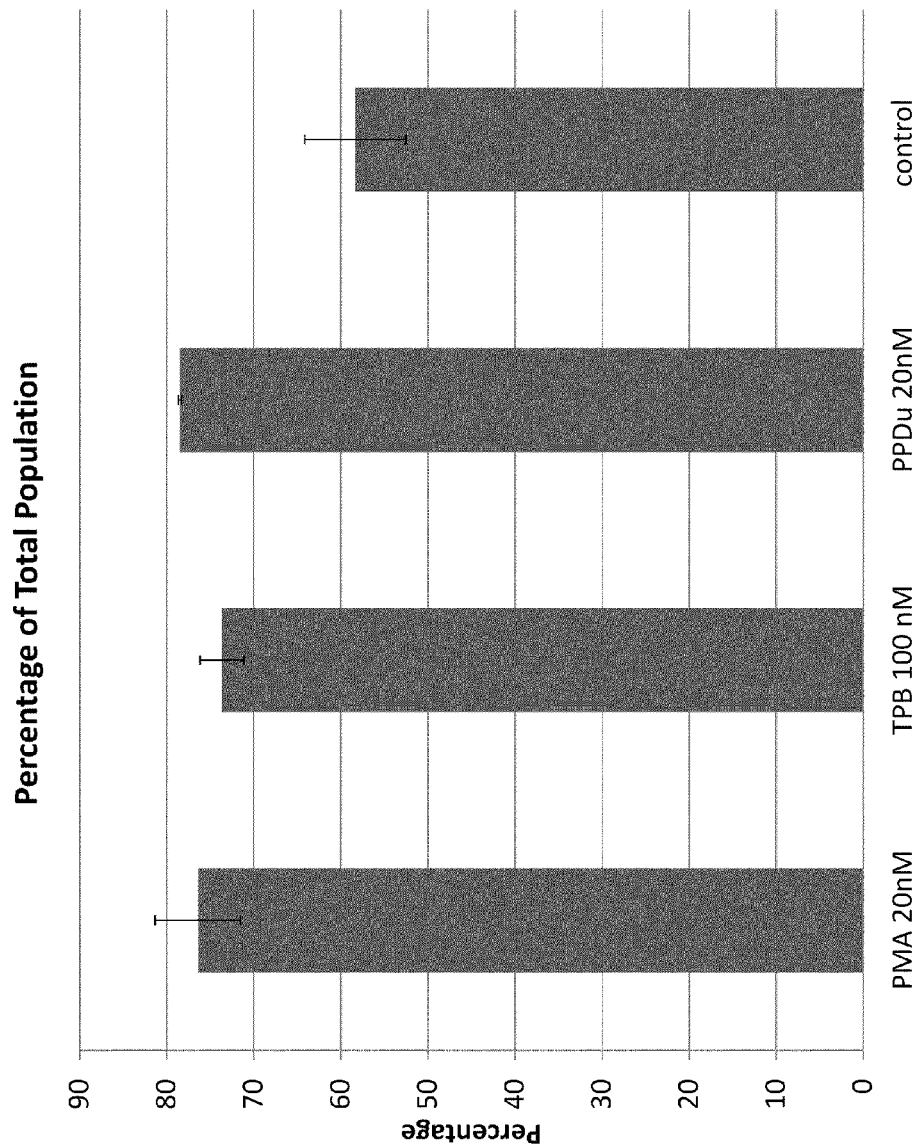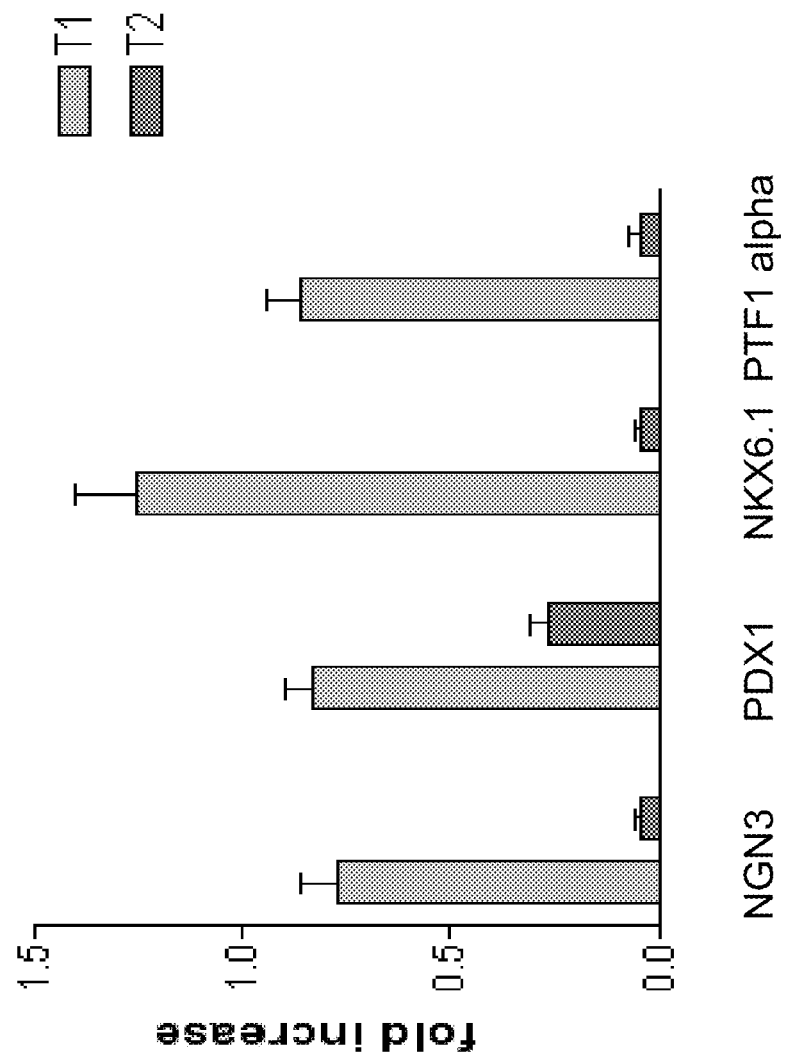
Figure 4

Figure 5

Figure 6

Figure 7

Figure 8

Figure 9

DIFFERENTIATION OF HUMAN EMBRYONIC STEM CELLS

This application claims the benefit of U.S. Provisional Application No. 61/289,671, filed on Dec. 23, 2009, which is incorporated by reference herein in its entirety.

FIELD OF THE INVENTION

The present invention provides methods to promote the differentiation of pluripotent stem cells into insulin producing cells. In particular, the present invention provides a method to produce cells expressing markers characteristic of the pancreatic endocrine lineage that co-express NKX6.1 and insulin and minimal amounts of glucagon.

BACKGROUND

Advances in cell-replacement therapy for Type I diabetes mellitus and a shortage of transplantable islets of Langerhans have focused interest on developing sources of insulin-producing cells, or β cells, appropriate for engraftment. One approach is the generation of functional β cells from pluripotent stem cells, such as, for example, embryonic stem cells.

In vertebrate embryonic development, a pluripotent cell gives rise to a group of cells comprising three germ layers (ectoderm, mesoderm, and endoderm) in a process known as gastrulation. Tissues such as, for example, thyroid, thymus, pancreas, gut, and liver, will develop from the endoderm, via an intermediate stage. The intermediate stage in this process is the formation of definitive endoderm. Definitive endoderm cells express a number of markers, such as, HNF3 beta, GATA4, MIXL1, CXCR4 and SOX17.

Formation of the pancreas arises from the differentiation of definitive endoderm into pancreatic endoderm. Cells of the pancreatic endoderm express the pancreatic-duodenal homeobox gene, PDX1. In the absence of PDX1, the pancreas fails to develop beyond the formation of ventral and dorsal buds. Thus, PDX1 expression marks a critical step in pancreatic organogenesis. The mature pancreas contains, among other cell types, exocrine tissue and endocrine tissue. Exocrine and endocrine tissues arise from the differentiation of pancreatic endoderm.

Cells bearing the features of islet cells have reportedly been derived from embryonic cells of the mouse. For example, Lumelsky et al. (Science 292:1389, 2001) report differentiation of mouse embryonic stem cells to insulin-secreting structures similar to pancreatic islets. Soria et al. (Diabetes 49:157, 2000) report that insulin-secreting cells derived from mouse embryonic stem cells normalize glycemia in streptozotocin-induced diabetic mice.

In one example, Hori et al. (PNAS 99:16105, 2002) disclose that treatment of mouse embryonic stem cells with inhibitors of phosphoinositide 3-kinase (LY294002) produced cells that resembled β cells.

In another example, Blyszczuk et al. (PNAS 100:998, 2003) reports the generation of insulin-producing cells from mouse embryonic stem cells constitutively expressing Pax4.

Micallef et al. reports that retinoic acid can regulate the commitment of embryonic stem cells to form PDX1 positive pancreatic endoderm. Retinoic acid is most effective at inducing Pdx1 expression when added to cultures at day 4 of embryonic stem cell differentiation during a period corresponding to the end of gastrulation in the embryo (Diabetes 54:301, 2005).

Miyazaki et al. reports a mouse embryonic stem cell line over-expressing Pdx1. Their results show that exogenous

Pdx1 expression clearly enhanced the expression of insulin, somatostatin, glucokinase, neurogenin3, p48, Pax6, and Hnf6 genes in the resulting differentiated cells (Diabetes 53:1030, 2004).

Skoudy et al. reports that activin A (a member of the TGF-β superfamily) upregulates the expression of exocrine pancreatic genes (p48 and amylase) and endocrine genes (Pdx1, insulin, and glucagon) in mouse embryonic stem cells. The maximal effect was observed using 1 nM activin A. They also observed that the expression level of insulin and Pdx1 mRNA was not affected by retinoic acid; however, 3 nM FGF7 treatment resulted in an increased level of the transcript for Pdx1 (Biochem. J. 379: 749, 2004).

Shiraki et al. studied the effects of growth factors that specifically enhance differentiation of embryonic stem cells into PDX1 positive cells. They observed that TGF-β reproducibly yielded a higher proportion of PDX1 positive cells (Genes Cells. 2005 June; 10(6): 503-16.).

Gordon et al. demonstrated the induction of brachyury [positive]/HNF3 beta [positive] endoderm cells from mouse embryonic stem cells in the absence of serum and in the presence of activin along with an inhibitor of Wnt signaling (US 2006/0003446A1).

Gordon et al. (PNAS, Vol 103, page 16806, 2006) states "Wnt and TGF-beta/nodal/activin signaling simultaneously were required for the generation of the anterior primitive streak".

However, the mouse model of embryonic stem cell development may not exactly mimic the developmental program in higher mammals, such as, for example, humans.

Thomson et al. isolated embryonic stem cells from human blastocysts (Science 282:114, 1998). Concurrently, Gearhart and coworkers derived human embryonic germ (hEG) cell lines from fetal gonadal tissue (Shamblott et al., Proc. Natl. Acad. Sci. USA 95:13726, 1998). Unlike mouse embryonic stem cells, which can be prevented from differentiating simply by culturing with Leukemia Inhibitory Factor (LIF), human embryonic stem cells must be maintained under very special conditions (U.S. Pat. No. 6,200,806; WO 99/20741; WO 01/51616).

D'Amour et al. describes the production of enriched cultures of human embryonic stem cell-derived definitive endoderm in the presence of a high concentration of activin and low serum (Nature Biotechnology 2005). Transplanting these cells under the kidney capsule of mice resulted in differentiation into more mature cells with characteristics of some endodermal organs. Human embryonic stem cell-derived definitive endoderm cells can be further differentiated into PDX1 positive cells after addition of FGF-10 (US 2005/0266554A1).

D'Amour et al. (Nature Biotechnology-24, 1392-1401 (2006)) states: "We have developed a differentiation process that converts human embryonic stem (hES) cells to endocrine cells capable of synthesizing the pancreatic hormones insulin, glucagon, somatostatin, pancreatic polypeptide and ghrelin. This process mimics in vivo pancreatic organogenesis by directing cells through stages resembling definitive endoderm, gut-tube endoderm, pancreatic endoderm and endocrine precursor en route to cells that express endocrine hormones".

In another example, Fisk et al. reports a system for producing pancreatic islet cells from human embryonic stem cells (US2006/0040387A1). In this case, the differentiation pathway was divided into three stages. Human embryonic stem cells were first differentiated to endoderm using a combination of sodium butyrate and activin A. The cells were then cultured with TGF-β antagonists such as Noggin in combi-

nation with EGF or betacellulin to generate PDX1 positive cells. The terminal differentiation was induced by nicotinamide.

In one example, Benvenistry et al. states: "We conclude that over-expression of PDX1 enhanced expression of pancreatic enriched genes, induction of insulin expression may require additional signals that are only present in vivo" (Benvenistry et al, Stem Cells 2006; 24:1923-1930).

In another example, Grapin-Botton et al. states: "Early activation of Ngn3 almost exclusively induced glucagon+ cells while depleting the pool of pancreas progenitors. As from E11.5, PDX-1 progenitors became competent to differentiate into insulin [positive] and PP [positive] cells" (Johansson K A et al, Developmental Cell 12, 457-465, March 2007).

For example, Diez et al. states; "At 9 and 10 weeks, most of the glucagon positive cells co-expressed insulin, although distinct insulin-only cells were clearly detectable at these stages. Cells co-expressing insulin and glucagon were observed during the whole period studies (9 to 21 weeks) but they represent merely a small fraction of the total insulin and glucagon expressing cells." (J Histochem Cytochem. 2009 September; 57(9):811-24. 2009 Apr. 13.)

In one example, Chen et al states ""(–)—indolactam V [(ILV)] activates protein kinase C signaling and directs the pancreatic specification of hESCs that have already been committed to the endoderm lineage . . . ILV and retinoic acid function through a related mechanism . . . ILV shows a stronger induction of PDX-1 expressing cells (percentage of cells expressing PDX-1) than does retinoic acid." (Nature Chemical Biology 5, 195-196 (April 2009) doi:10.1038/nchembio0409-195).

Lyttle et al states: "NKX6-1 co-localised only with insulin cells, indicating that NKX6-1 is exclusively involved in human beta cell development." (Diabetologia 2008 July: 51(7):1169-80, 2008).

Therefore, there still remains a significant need to develop in vitro methods to generate a functional insulin expressing cell, that more closely resemble a β cell. The present invention takes an alternative approach to improve the efficiency of differentiating human embryonic stem cells toward insulin expressing cells, by generating a population of cells expressing markers characteristic of the pancreatic endocrine lineage that co-express NKX6.1 and insulin and minimal amounts of glucagon.

SUMMARY

In one embodiment, the present invention provides a population of cells expressing markers characteristic of the pancreatic endocrine lineage that co-express NKX6.1 and insulin and minimal amounts of glucagon.

In one embodiment, the present invention provides a method to differentiate a population of pluripotent stem cells into a population of cells expressing markers characteristic of the pancreatic endocrine lineage that co-express NKX6.1 and insulin and minimal amounts of glucagon, comprising the steps of:

a. Culturing the pluripotent stem cells,

b. Differentiating the pluripotent stem cells into cells expressing markers characteristic of the definitive endoderm lineage,

c. Differentiating the cells expressing markers characteristic of the definitive endoderm lineage into cells expressing markers characteristic of the pancreatic endoderm lineage, and

d. Differentiating the cells expressing markers characteristic of the pancreatic endoderm lineage into cells expressing

markers characteristic of the pancreatic endocrine lineage that co-express NKX6.1 and insulin and minimal amounts of glucagon, by treating the cells expressing markers characteristic of the pancreatic endoderm lineage with medium supplemented with a protein kinase C activator.

BRIEF DESCRIPTION OF THE DRAWINGS

FIG. 1 shows the effect of TPB treatment on the expression of insulin and glucagon in the cells of the present invention. Panels a and b show the expression of insulin and glucagon respectively, in cells treated with TPB. Control populations of cells are shown in panels c and d.

FIG. 2 shows the effect of various concentrations of TPB on the expression of insulin and glucagon in cells treated according to the methods of the present invention. Panels a through d show the expression of insulin and glucagon in populations of cells treated with TPB at the doses indicated.

FIG. 3 shows the effect of a protein kinase C inhibitor on the expression of insulin and glucagon in cells treated according to the methods of the present invention. Panel a depicts the expression of insulin and glucagon in cells treated with TPB, and Panel c depicts the corresponding DAPI staining). Panel b depicts the expression of insulin and glucagon in cells treated with TPB and GÖ 6976, and Panel d depicts the corresponding DAPI staining).

FIG. 4 shows the effect of various protein kinase C activators on the expression of insulin in cells treated according to the methods of the present invention. Panel a shows the expression of insulin in cells treated with TPB. Panel b shows the expression of insulin in cells treated with ILV. Panel c shows the expression of insulin in cells treated with PMA.

FIG. 5 shows the expression of markers characteristic of the pancreatic endocrine lineage in cells treated according to the methods of the present invention. The panels depict the expression of insulin and NKX6.1 (panel a), insulin and PDX1 (panel b), insulin and NEUROD1 (panel c), insulin and somatostatin (panel d), and insulin and ghrelin (panel e).

FIG. 6 shows the expression of insulin and glucagon in cells treated according to the methods of the present invention. Panels a to c show insulin expression (panel a), glucagon expression (panel b) and DAPI staining (panel c) in cells treated with DMEM-High glucose+1% B27+50 ng/ml FGF7+0.25 µM Cyclopamine–KAAD+2 µM Retinoic acid (RA)+100 ng/ml of Noggin+20 ng/ml Activin A+a p38 kinase inhibitor (disclosed in U.S. Pat. No. 6,214,830, at 2.5 µM) for four days (Stage 3, Treatment 8, Example 2). Panels d to f show insulin expression (panel d), glucagon expression (panel e) and DAPI staining (panel f) in cells treated with DMEM-High glucose+1% B27+0.25 µM Cyclopamine–KAAD+2 µM Retinoic acid (RA)+100 ng/ml of Noggin for four days (Stage 3, Treatment 9, Example 2).

FIG. 7 shows human C-peptide was detected in (SCID)— beige (Bg) mice four, eight and twelve weeks after receiving the cells of the present invention, following a glucose challenge.

FIG. 8 shows the percentage of cells co-expressing PDX1 and NKX6.1 obtained following treatment of various protein kinase C inhibitors, at the concentrations indicated.

FIG. 9 shows the expression of NGN3, PDX1, NKX6.1 and PTF1 alpha in cells treated according to the methods described in Example 6.

DETAILED DESCRIPTION

For clarity of disclosure, and not by way of limitation, the detailed description of the invention is divided into the fol-

lowing subsections that describe or illustrate certain features, embodiments or applications of the present invention.

DEFINITIONS

Stem cells are undifferentiated cells defined by their ability at the single cell level to both self-renew and differentiate to produce progeny cells, including self-renewing progenitors, non-renewing progenitors, and terminally differentiated cells. Stem cells are also characterized by their ability to differentiate in vitro into functional cells of various cell lineages from multiple germ layers (endoderm, mesoderm and ectoderm), as well as to give rise to tissues of multiple germ layers following transplantation and to contribute substantially to most, if not all, tissues following injection into blastocysts.

Stem cells are classified by their developmental potential as: (1) totipotent, meaning able to give rise to all embryonic and extraembryonic cell types; (2) pluripotent, meaning able to give rise to all embryonic cell types; (3) multipotent, meaning able to give rise to a subset of cell lineages but all within a particular tissue, organ, or physiological system (for example, hematopoietic stem cells (HSC) can produce progeny that include HSC (self-renewal), blood cell restricted oligopotent progenitors, and all cell types and elements (e.g., platelets) that are normal components of the blood); (4) oligopotent, meaning able to give rise to a more restricted subset of cell lineages than multipotent stem cells; and (5) unipotent, meaning able to give rise to a single cell lineage (e.g., spermatogenic stem cells).

Differentiation is the process by which an unspecialized ("uncommitted") or less specialized cell acquires the features of a specialized cell such as, for example, a nerve cell or a muscle cell. A differentiated or differentiation-induced cell is one that has taken on a more specialized ("committed") position within the lineage of a cell. The term "committed", when applied to the process of differentiation, refers to a cell that has proceeded in the differentiation pathway to a point where, under normal circumstances, it will continue to differentiate into a specific cell type or subset of cell types, and cannot, under normal circumstances, differentiate into a different cell type or revert to a less differentiated cell type. De-differentiation refers to the process by which a cell reverts to a less specialized (or committed) position within the lineage of a cell. As used herein, the lineage of a cell defines the heredity of the cell, i.e., which cells it came from and what cells it can give rise to. The lineage of a cell places the cell within a hereditary scheme of development and differentiation. A lineage-specific marker refers to a characteristic specifically associated with the phenotype of cells of a lineage of interest and can be used to assess the differentiation of an uncommitted cell to the lineage of interest.

"Cells expressing markers characteristic of the definitive endoderm lineage", or "Stage 1 cells", or "Stage 1", as used herein, refers to cells expressing at least one of the following markers: SOX17, GATA4, HNF3 beta, GSC, CER1, Nodal, FGF8, Brachyury, Mix-like homeobox protein, FGF4 CD48, eomesodermin (EOMES), DKK4, FGF17, GATA6, CXCR4, C-Kit, CD99, or OTX2. Cells expressing markers characteristic of the definitive endoderm lineage include primitive streak precursor cells, primitive streak cells, mesendoderm cells and definitive endoderm cells.

"Cells expressing markers characteristic of the pancreatic endoderm lineage", as used herein, refers to cells expressing at least one of the following markers: PDX1, NKX6.1, HNF1 beta, PTF1 alpha, HNF6, HNF4 alpha, SOX9, HB9 or PROX1. Cells expressing markers characteristic of the pan-

creatic endoderm lineage include pancreatic endoderm cells, primitive gut tube cells, and posterior foregut cells.

"Definitive endoderm", as used herein, refers to cells which bear the characteristics of cells arising from the epiblast during gastrulation and which form the gastrointestinal tract and its derivatives. Definitive endoderm cells express the following markers: HNF3 beta, GATA4, SOX17, Cerberus, OTX2, goosecoid, C-Kit, CD99, and MIXL1.

"Markers", as used herein, are nucleic acid or polypeptide molecules that are differentially expressed in a cell of interest. In this context, differential expression means an increased level for a positive marker and a decreased level for a negative marker. The detectable level of the marker nucleic acid or polypeptide is sufficiently higher or lower in the cells of interest compared to other cells, such that the cell of interest can be identified and distinguished from other cells using any of a variety of methods known in the art.

"Pancreatic endocrine cell", or "Pancreatic hormone expressing cell", or "Cells expressing markers characteristic of the pancreatic endocrine lineage" as used herein, refers to a cell capable of expressing at least one of the following hormones: insulin, glucagon, somatostatin, and pancreatic polypeptide.

Isolation, Expansion and Culture of Pluripotent Stem Cells

Characterization of Pluripotent Stem Cells

Pluripotent stem cells may express one or more of the stage-specific embryonic antigens (SSEA) 3 and 4, and markers detectable using antibodies designated Tra-1-60 and Tra-1-81 (Thomson et al., Science 282:1145, 1998). Differentiation of pluripotent stem cells in vitro results in the loss of SSEA-4, Tra 1-60, and Tra 1-81 expression (if present) and increased expression of SSEA-1. Undifferentiated pluripotent stem cells typically have alkaline phosphatase activity, which can be detected by fixing the cells with 4% paraformaldehyde, and then developing with Vector Red as a substrate, as described by the manufacturer (Vector Laboratories, Burlingame Calif.). Undifferentiated pluripotent stem cells also typically express OCT4 and TERT, as detected by RT-PCR.

Another desirable phenotype of propagated pluripotent stem cells is a potential to differentiate into cells of all three germinal layers: endoderm, mesoderm, and ectoderm tissues. Pluripotency of pluripotent stem cells can be confirmed, for example, by injecting cells into severe combined immunodeficient (SCID) mice, fixing the teratomas that form using 4% paraformaldehyde, and then examining them histologically for evidence of cell types from the three germ layers. Alternatively, pluripotency may be determined by the creation of embryoid bodies and assessing the embryoid bodies for the presence of markers associated with the three germinal layers.

Propagated pluripotent stem cell lines may be karyotyped using a standard G-banding technique and compared to published karyotypes of the corresponding primate species. It is desirable to obtain cells that have a "normal karyotype," which means that the cells are euploid, wherein all human chromosomes are present and not noticeably altered.

Sources of Pluripotent Stem Cells

The types of pluripotent stem cells that may be used include established lines of pluripotent cells derived from tissue formed after gestation, including pre-embryonic tissue (such as, for example, a blastocyst), embryonic tissue, or fetal

tissue taken any time during gestation, typically but not necessarily before approximately 10 to 12 weeks gestation. Non-limiting examples are established lines of human embryonic stem cells or human embryonic germ cells, such as, for example the human embryonic stem cell lines H1, H7, and H9 (WiCell). Also contemplated is use of the compositions of this disclosure during the initial establishment or stabilization of such cells, in which case the source cells would be primary pluripotent cells taken directly from the source tissues. Also suitable are cells taken from a pluripotent stem cell population already cultured in the absence of feeder cells. Also suitable are mutant human embryonic stem cell lines, such as, for example, BG01v (BresaGen, Athens, Ga.).

In one embodiment, human embryonic stem cells are prepared as described by Thomson et al. (U.S. Pat. No. 5,843, 780; Science 282:1145, 1998; Curr. Top. Dev. Biol. 38:133 ff., 1998; Proc. Natl. Acad. Sci. U.S.A. 92:7844, 1995).

Culture of Pluripotent Stem Cells

In one embodiment, pluripotent stem cells are typically cultured on a layer of feeder cells that support the pluripotent stem cells in various ways. Alternatively, pluripotent stem cells are cultured in a culture system that is essentially free of feeder cells, but nonetheless supports proliferation of pluripotent stem cells without undergoing substantial differentiation. The growth of pluripotent stem cells in feeder-free culture without differentiation is supported using a medium conditioned by culturing previously with another cell type. Alternatively, the growth of pluripotent stem cells in feeder-free culture without differentiation is supported using a chemically defined medium.

For example, Reubinoff et al (Nature Biotechnology 18: 399-404 (2000)) and Thompson et al (Science 6 Nov. 1998: Vol. 282. no. 5391, pp. 1145-1147) disclose the culture of pluripotent stem cell lines from human blastocysts using a mouse embryonic fibroblast feeder cell layer.

Richards et al, (Stem Cells 21: 546-556, 2003) evaluated a panel of 11 different human adult, fetal and neonatal feeder cell layers for their ability to support human pluripotent stem cell culture. Richards et al, states: "human embryonic stem cell lines cultured on adult skin fibroblast feeders retain human embryonic stem cell morphology and remain pluripotent".

US20020072117 discloses cell lines that produce media that support the growth of primate pluripotent stem cells in feeder-free culture. The cell lines employed are mesenchymal and fibroblast-like cell lines obtained from embryonic tissue or differentiated from embryonic stem cells. US20020072117 also discloses the use of the cell lines as a primary feeder cell layer.

In another example, Wang et al (Stem Cells 23: 1221-1227, 2005) discloses methods for the long-term growth of human pluripotent stem cells on feeder cell layers derived from human embryonic stem cells.

In another example, Stojkovic et al (Stem Cells 2005 23:306-314, 2005) disclose a feeder cell system derived from the spontaneous differentiation of human embryonic stem cells.

In a further example, Miyamoto et al (Stem Cells 22: 433-440, 2004) disclose a source of feeder cells obtained from human placenta.

Amit et al (Biol. Reprod 68: 2150-2156, 2003) discloses a feeder cell layer derived from human foreskin.

In another example, Inzunza et al (Stem Cells 23: 544-549, 2005) disclose a feeder cell layer from human postnatal foreskin fibroblasts.

U.S. Pat. No. 6,642,048 discloses media that support the growth of primate pluripotent stem (pPS) cells in feeder-free culture, and cell lines useful for production of such media. U.S. Pat. No. 6,642,048 states: "This invention includes mesenchymal and fibroblast-like cell lines obtained from embryonic tissue or differentiated from embryonic stem cells. Methods for deriving such cell lines, processing media, and growing stem cells using the conditioned media are described and illustrated in this disclosure."

In another example, WO2005014799 discloses conditioned medium for the maintenance, proliferation and differentiation of mammalian cells. WO2005014799 states: "The culture medium produced in accordance with the present invention is conditioned by the cell secretion activity of murine cells; in particular, those differentiated and immortalized transgenic hepatocytes, named MMH (Met Murine Hepatocyte)."

In another example, Xu et al (Stem Cells 22: 972-980, 2004) discloses conditioned medium obtained from human embryonic stem cell derivatives that have been genetically modified to over express human telomerase reverse transcriptase.

In another example, US20070010011 discloses a chemically defined culture medium for the maintenance of pluripotent stem cells.

An alternative culture system employs serum-free medium supplemented with growth factors capable of promoting the proliferation of embryonic stem cells. For example, Cheon et al (BioReprod DOI: 10.1095/biolreprod.105.046870, Oct. 19, 2005) disclose a feeder-free, serum-free culture system in which embryonic stem cells are maintained in unconditioned serum replacement (SR) medium supplemented with different growth factors capable of triggering embryonic stem cell self-renewal.

In another example, Levenstein et al (Stem Cells 24: 568-574, 2006) disclose methods for the long-term culture of human embryonic stem cells in the absence of fibroblasts or conditioned medium, using media supplemented with bFGF.

In another example, US20050148070 discloses a method of culturing human embryonic stem cells in defined media without serum and without fibroblast feeder cells, the method comprising: culturing the stem cells in a culture medium containing albumin, amino acids, vitamins, minerals, at least one transferrin or transferrin substitute, at least one insulin or insulin substitute, the culture medium essentially free of mammalian fetal serum and containing at least about 100 ng/ml of a fibroblast growth factor capable of activating a fibroblast growth factor signaling receptor, wherein the growth factor is supplied from a source other than just a fibroblast feeder layer, the medium supported the proliferation of stem cells in an undifferentiated state without feeder cells or conditioned medium.

In another example, US20050233446 discloses a defined media useful in culturing stem cells, including undifferentiated primate primordial stem cells. In solution, the media is substantially isotonic as compared to the stem cells being cultured. In a given culture, the particular medium comprises a base medium and an amount of each of bFGF, insulin, and ascorbic acid necessary to support substantially undifferentiated growth of the primordial stem cells.

In another example, U.S. Pat. No. 6,800,480 states "In one embodiment, a cell culture medium for growing primate-derived primordial stem cells in a substantially undifferentiated state is provided which includes a low osmotic pressure, low endotoxin basic medium that is effective to support the growth of primate-derived primordial stem cells. The basic medium is combined with a nutrient serum effective to sup-

port the growth of primate-derived primordial stem cells and a substrate selected from the group consisting of feeder cells and an extracellular matrix component derived from feeder cells. The medium further includes non-essential amino acids, an anti-oxidant, and a first growth factor selected from the group consisting of nucleosides and a pyruvate salt."

In another example, US20050244962 states: "In one aspect the invention provides a method of culturing primate embryonic stem cells. One cultures the stem cells in a culture essentially free of mammalian fetal serum (preferably also essentially free of any animal serum) and in the presence of fibroblast growth factor that is supplied from a source other than just a fibroblast feeder layer. In a preferred form, the fibroblast feeder layer, previously required to sustain a stem cell culture, is rendered unnecessary by the addition of sufficient fibroblast growth factor."

In a further example, WO2005065354 discloses a defined, isotonic culture medium that is essentially feeder-free and serum-free, comprising: a. a basal medium; b. an amount of bFGF sufficient to support growth of substantially undifferentiated mammalian stem cells; c. an amount of insulin sufficient to support growth of substantially undifferentiated mammalian stem cells; and d. an amount of ascorbic acid sufficient to support growth of substantially undifferentiated mammalian stem cells.

In another example, WO2005086845 discloses a method for maintenance of an undifferentiated stem cell, said method comprising exposing a stem cell to a member of the transforming growth factor-beta (TGF-β) family of proteins, a member of the fibroblast growth factor (FGF) family of proteins, or nicotinamide (NIC) in an amount sufficient to maintain the cell in an undifferentiated state for a sufficient amount of time to achieve a desired result.

The pluripotent stem cells may be plated onto a suitable culture substrate. In one embodiment, the suitable culture substrate is an extracellular matrix component, such as, for example, those derived from basement membrane or that may form part of adhesion molecule receptor-ligand couplings. In one embodiment, a the suitable culture substrate is MATRIGEL® (Becton Dickenson). MATRIGEL® is a soluble preparation from Engelbreth-Holm Swarm tumor cells that gels at room temperature to form a reconstituted basement membrane.

Other extracellular matrix components and component mixtures are suitable as an alternative. Depending on the cell type being proliferated, this may include laminin, fibronectin, proteoglycan, entactin, heparan sulfate, and the like, alone or in various combinations.

The pluripotent stem cells may be plated onto the substrate in a suitable distribution and in the presence of a medium that promotes cell survival, propagation, and retention of the desirable characteristics. All these characteristics benefit from careful attention to the seeding distribution and can readily be determined by one of skill in the art.

Suitable culture media may be made from the following components, such as, for example, Dulbecco's modified Eagle's medium (DMEM), Gibco #11965-092; Knockout Dulbecco's modified Eagle's medium (KO DMEM), Gibco #10829-018; Ham's F12/50% DMEM basal medium; 200 mM L-glutamine, Gibco #15039-027; non-essential amino acid solution, Gibco 11140-050; β-mercaptoethanol, Sigma #M7522; human recombinant basic fibroblast growth factor (bFGF), Gibco #13256-029.

Formation of Cells Expressing Markers
Characteristic of the Pancreatic Endocrine Lineage
from Pluripotent Stem Cells

In one embodiment, the present invention provides a method for producing cells expressing markers characteristic of the pancreatic endoderm lineage from pluripotent stem cells, comprising the steps of:
    a. Culturing pluripotent stem cells,
    b. Differentiating the pluripotent stem cells into cells expressing markers characteristic of the definitive endoderm lineage,
    c. Differentiating the cells expressing markers characteristic of the definitive endoderm lineage into cells expressing markers characteristic of the pancreatic endoderm lineage, and
    d. Differentiating the cells expressing markers characteristic of the pancreatic endoderm lineage into cells expressing markers characteristic of the pancreatic endocrine lineage.

In one aspect of the present invention, the cells expressing markers characteristic of the pancreatic endocrine lineage co-express NKX6.1 and insulin and minimal amounts of glucagon.

Differentiation of Pluripotent Stem Cells into Cells
Expressing Markers Characteristic of the Definitive
Endoderm Lineage

Formation of cells expressing markers characteristic of the definitive endoderm lineage may be determined by testing for the presence of the markers before and after following a particular protocol. Pluripotent stem cells typically minimal amounts of such markers. Thus, differentiation of pluripotent cells is detected when cells begin to express them.

Pluripotent stem cells may be differentiated into cells expressing markers characteristic of the definitive endoderm lineage by any method in the art or by any method proposed in this invention.

For example, pluripotent stem cells may be differentiated into cells expressing markers characteristic of the definitive endoderm lineage according to the methods disclosed in D'Amour et al, Nature Biotechnology 23, 1534-1541 (2005).

For example, pluripotent stem cells may be differentiated into cells expressing markers characteristic of the definitive endoderm lineage according to the methods disclosed in Shinozaki et al, Development 131, 1651-1662 (2004).

For example, pluripotent stem cells may be differentiated into cells expressing markers characteristic of the definitive endoderm lineage according to the methods disclosed in McLean et al, Stem Cells 25, 29-38 (2007).

For example, pluripotent stem cells may be differentiated into cells expressing markers characteristic of the definitive endoderm lineage according to the methods disclosed in D'Amour et al, Nature Biotechnology 24, 1392-1401 (2006).

For example, pluripotent stem cells may be differentiated into cells expressing markers characteristic of the definitive endoderm lineage by culturing the pluripotent stem cells in medium containing activin A in the absence of serum, then culturing the cells with activin A and serum, and then culturing the cells with activin A and serum of a different concentration. An example of this method is disclosed in Nature Biotechnology 23, 1534-1541 (2005).

For example, pluripotent stem cells may be differentiated into cells expressing markers characteristic of the definitive endoderm lineage by culturing the pluripotent stem cells in medium containing activin A in the absence of serum, then

culturing the cells with activin A with serum of another concentration. An example of this method is disclosed in D'Amour et al, Nature Biotechnology, 2005.

For example, pluripotent stem cells may be differentiated into cells expressing markers characteristic of the definitive endoderm lineage by culturing the pluripotent stem cells in medium containing activin A and a Wnt ligand in the absence of serum, then removing the Wnt ligand and culturing the cells with activin A with serum. An example of this method is disclosed in Nature Biotechnology 24, 1392-1401 (2006).

For example, pluripotent stem cells may be differentiated into cells expressing markers characteristic of the definitive endoderm lineage by treating the pluripotent stem cells according to the methods disclosed in U.S. patent application Ser. No. 11/736,908, assigned to LifeScan, Inc.

For example, pluripotent stem cells may be differentiated into cells expressing markers characteristic of the definitive endoderm lineage by treating the pluripotent stem cells according to the methods disclosed in U.S. patent application Ser. No. 11/779,311, assigned to LifeScan, Inc.

For example, pluripotent stem cells may be differentiated into cells expressing markers characteristic of the definitive endoderm lineage by treating the pluripotent stem cells according to the methods disclosed in U.S. patent application Ser. No. 60/990,529.

For example, pluripotent stem cells may be differentiated into cells expressing markers characteristic of the definitive endoderm lineage by treating the pluripotent stem cells according to the methods disclosed in U.S. patent application Ser. No. 61/076,889.

For example, pluripotent stem cells may be differentiated into cells expressing markers characteristic of the definitive endoderm lineage by treating the pluripotent stem cells according to the methods disclosed in U.S. patent application Ser. No. 61/076,900.

For example, pluripotent stem cells may be differentiated into cells expressing markers characteristic of the definitive endoderm lineage by treating the pluripotent stem cells according to the methods disclosed in U.S. patent application Ser. No. 61/076,908.

For example, pluripotent stem cells may be differentiated into cells expressing markers characteristic of the definitive endoderm lineage by treating the pluripotent stem cells according to the methods disclosed in U.S. patent application Ser. No. 61/076,915.

Differentiation of Cells Expressing Markers Characteristic of the Definitive Endoderm Lineage into Cells Expressing Markers Characteristic of the Pancreatic Endoderm Lineage

Cells expressing markers characteristic of the definitive endoderm lineage may be differentiated into cells expressing markers characteristic of the pancreatic endoderm lineage by any method in the art or by any method proposed in this invention.

For example, cells expressing markers characteristic of the definitive endoderm lineage may be differentiated into cells expressing markers characteristic of the pancreatic endoderm lineage according to the methods disclosed in D'Amour et al., Nature Biotechnol. 24:1392-1401, 2006.

In one embodiment, the cells expressing markers characteristic of the pancreatic endoderm lineage co-express PDX1, NKX6.1, but minimal amounts of CDX2 and NGN3.

In one embodiment, cells expressing markers characteristic of the definitive endoderm lineage are differentiated into cells expressing markers characteristic of the pancreatic

endoderm lineage that co-express PDX1, NKX6.1, but minimal amounts of CDX2 and NGN3, by culturing the cells expressing markers characteristic of the definitive endoderm lineage in a first medium supplemented with FGF7, followed by culturing the cells in a second medium supplemented with FGF7, a factor capable of inhibiting BMP, a TGFβ receptor agonist, retinoic acid, and a hedgehog signaling pathway inhibitor.

In one embodiment, FGF7 may be used at a concentration from about 50 pg/ml to about 50 μg/ml. In one embodiment, FGF7 is used at a concentration of 50 ng/ml.

In one embodiment, the factor capable of inhibiting BMP is noggin. Noggin may be used at a concentration from about 500 ng/ml to about 500 μg/ml. In one embodiment, noggin is used at a concentration of 100 ng/ml.

In one embodiment, the TGFβ receptor agonist is selected from the group consisting of activin A, activin B, TGFβ-I, TGFβ-II, GDF-8, and GDF-11.

Activin A may be used at a concentration from about 2 ng/ml to 100 ng/ml. In one embodiment, activin A is used at a concentration of 20 ng/ml. In an alternate embodiment, activin A is used at a concentration of 50 ng/ml.

Activin B may be used at a concentration from about 2 ng/ml to 100 ng/ml. In one embodiment, activin B is used at a concentration of 20 ng/ml. In an alternate embodiment, activin B is used at a concentration of 50 ng/ml.

TGFβ-I may be used at a concentration from about 2 ng/ml to 100 ng/ml. In one embodiment, TGFβ-I is used at a concentration of 20 ng/ml. In an alternate embodiment, TGFβ-I is used at a concentration of 50 ng/ml.

TGFβ-II may be used at a concentration from about 2 ng/ml to 100 ng/ml. In one embodiment, TGFβ-II is used at a concentration of 20 ng/ml. In an alternate embodiment, TGFβ-II is used at a concentration of 50 ng/ml.

GDF-8 may be used at a concentration from about 2 ng/ml to 100 ng/ml. In one embodiment, GDF-8 is used at a concentration of 20 ng/ml. In an alternate embodiment, GDF-8 is used at a concentration of 50 ng/ml.

GDF-11 may be used at a concentration from about 2 ng/ml to 100 ng/ml. In one embodiment, GDF-11 is used at a concentration of 20 ng/ml. In an alternate embodiment, GDF-11 is used at a concentration of 50 ng/ml.

Retinoic acid may be used at a concentration from about 1 nM to about 1 mM. In one embodiment, retinoic acid is used at a concentration of 1 μM.

In one embodiment, the hedgehog signaling pathway inhibitor is cyclopamine-KAAD. Cyclopamine-KAAD may be used at a concentration from about 0.025 μM to about 2.5 μM. In one embodiment, cyclopamine-KAAD is used at a concentration of 0.25 μM.

The efficiency of differentiation may be determined by exposing a treated cell population to an agent (such as an antibody) that specifically recognizes a protein marker expressed by cells expressing markers characteristic of the definitive endoderm lineage.

Methods for assessing expression of protein and nucleic acid markers in cultured or isolated cells are standard in the art. These include quantitative reverse transcriptase polymerase chain reaction (RT-PCR), Northern blots, in situ hybridization (see, e.g., Current Protocols in Molecular Biology (Ausubel et al., eds. 2001 supplement)), and immunoassays such as immunohistochemical analysis of sectioned material, Western blotting, and for markers that are accessible in intact cells, flow cytometry analysis (FACS) (see, e.g., Harlow and Lane, Using Antibodies: A Laboratory Manual, N.Y.: Cold Spring Harbor Laboratory Press (1998)).

Characteristics of pluripotent stem cells are well known to those skilled in the art, and additional characteristics of pluripotent stem cells continue to be identified. Pluripotent stem cell markers include, for example, the expression of one or more of the following: ABCG2, cripto, FOXD3, CONNEXIN43, CONNEXIN45, OCT4, SOX2, Nanog, hTERT, UTF1, ZFP42, SSEA-3, SSEA-4, Tra 1-60, Tra 1-81.

After treating pluripotent stem cells with the methods of the present invention, the differentiated cells may be purified by exposing a treated cell population to an agent (such as an antibody) that specifically recognizes a protein marker, such as CXCR4, expressed by cells expressing markers characteristic of the definitive endoderm lineage.

Pluripotent stem cells suitable for use in the present invention include, for example, the human embryonic stem cell line H9 (NIH code: WA09), the human embryonic stem cell line H1 (NIH code: WA01), the human embryonic stem cell line H7 (NIH code: WA07), and the human embryonic stem cell line SA002 (Cellartis, Sweden). Also suitable for use in the present invention are cells that express at least one of the following markers characteristic of pluripotent cells: ABCG2, cripto, CD9, FOXD3, CONNEXIN43, CONNEXIN45, OCT4, SOX2, Nanog, hTERT, UTF1, ZFP42, SSEA-3, SSEA-4, Tra 1-60, and Tra 1-81.

Markers characteristic of the definitive endoderm lineage are selected from the group consisting of SOX17, GATA4, HNF3 beta, GSC, CER1, Nodal, FGF8, Brachyury, Mix-like homeobox protein, FGF4, CD48, eomesodermin (EOMES), DKK4, FGF17, GATA6, CXCR4, C-Kit, CD99, and OTX2. Suitable for use in the present invention is a cell that expresses at least one of the markers characteristic of the definitive endoderm lineage. In one aspect of the present invention, a cell expressing markers characteristic of the definitive endoderm lineage is a primitive streak precursor cell. In an alternate aspect, a cell expressing markers characteristic of the definitive endoderm lineage is a mesendoderm cell. In an alternate aspect, a cell expressing markers characteristic of the definitive endoderm lineage is a definitive endoderm cell.

Markers characteristic of the pancreatic endoderm lineage are selected from the group consisting of PDX1, NKX6.1, HNF1 beta, PTF1 alpha, HNF6, HNF4 alpha, SOX9, HB9 and PROX1. Suitable for use in the present invention is a cell that expresses at least one of the markers characteristic of the pancreatic endoderm lineage. In one aspect of the present invention, a cell expressing markers characteristic of the pancreatic endoderm lineage is a pancreatic endoderm cell.

Differentiation of Cells Expressing Markers Characteristic of the Pancreatic Endoderm Lineage into Cells Expressing Markers of the Pancreatic Endocrine Lineage

In one embodiment, cells expressing markers characteristic of the pancreatic endoderm lineage are further differentiated into cells expressing markers characteristic of the pancreatic endocrine lineage.

In one embodiment, the cells expressing markers characteristic of the pancreatic endoderm lineage co-express PDX1, NKX6.1, but minimal amounts of CDX2 and NGN3.

In one embodiment, the cells expressing markers characteristic of the pancreatic endocrine lineage co-express NKX6.1 and insulin and minimal amounts of glucagon.

In one embodiment, cells expressing markers characteristic of the pancreatic endoderm lineage are differentiated into cells expressing markers characteristic of the pancreatic endocrine lineage that co-express NKX6.1 and insulin and minimal amounts of glucagon, by culturing the cells express-

ing markers characteristic of the pancreatic endoderm lineage in a medium supplemented with a factor capable of inhibiting BMP, a TGFβ receptor signaling inhibitor, and a protein kinase C activator.

In one embodiment, the factor capable of inhibiting BMP is noggin. Noggin may be used at a concentration from about 500 ng/ml to about 500 μg/ml. In one embodiment, noggin is used at a concentration of 100 ng/ml.

In one embodiment, the TGFβ receptor signaling inhibitor is an inhibitor of ALK5. In one embodiment, the inhibitor of ALK5 is ALK5 inhibitor II. The ALK5 inhibitor II may be used at a concentration from about 0.1 μM to about 10 μM. In one embodiment, ALK5 inhibitor II is used at a concentration of 1 μM.

In one embodiment, the protein kinase C activator is selected from the group consisting of (2S,5S)-(E,E)-8-(5-(4-(Trifluoromethyl)phenyl)-2,4-pentadienoylamino)benzolactam, Indolactam V, and phorbol-12-myristate-13-acetate. In one embodiment, the protein kinase C activator is (2S,5S)-(E,E)-8-(5-(4-(Trifluoromethyl)phenyl)-2,4-pentadiemoylamino)benzolactam. (2S,5S)-(E,E)-8-(5-(4-(Trifluoromethyl)phenyl)-2,4-pentadienoylamino)benzolactam may be used at a concentration from about 20 nM to about 500 nM. (2S,5S)-(E,E)-8-(5-(4-(Trifluoromethyl)phenyl)-2,4-pentadienoylamino)benzolactam, Indolactam V, and phorbol-12-myristate-13-acetate is referred to herein as "TPB".

Markers characteristic of the pancreatic endocrine lineage are selected from the group consisting of NEUROD, ISL1, PDX1, NKX6.1, NKX2.2, PAX4, and PAX6. In one embodiment, the cells expressing markers characteristic of the pancreatic endocrine lineage co-express NKX6.1 and insulin and minimal amounts of glucagon.

Therapies

In one aspect, the present invention provides a method for treating a patient suffering from, or at risk of developing, Type1 diabetes. In one embodiment, the method involves culturing pluripotent stem cells, differentiating the pluripotent stem cells in vitro into cells expressing markers characteristic of the pancreatic endocrine lineage, and implanting the cells expressing markers characteristic of the pancreatic endocrine lineage into a patient.

In yet another aspect, this invention provides a method for treating a patient suffering from, or at risk of developing, Type 2 diabetes. In one embodiment, the method involves culturing pluripotent stem cells, differentiating the pluripotent stem cells in vitro into cells expressing markers characteristic of the pancreatic endocrine lineage, and implanting the cells expressing markers characteristic of the pancreatic endocrine lineage into a patient.

If appropriate, the patient can be further treated with pharmaceutical agents or bioactives that facilitate the survival and function of the transplanted cells. These agents may include, for example, insulin, members of the TGF-β family, including TGF-β1, 2, and 3, bone morphogenic proteins (BMP-2, -3, -4, -5, -6, -7, -11, -12, and -13), fibroblast growth factors-1 and -2, platelet-derived growth factor-AA, and -BB, platelet rich plasma, insulin growth factor (IGF-I, II) growth differentiation factor (GDF-5, -6, -7, -8, -10, -15), vascular endothelial cell-derived growth factor (VEGF), pleiotrophin, endothelin, among others. Other pharmaceutical compounds can include, for example, nicotinamide, glucagon like peptide-I (GLP-1) and II, GLP-1 and 2 mimetibody, Exendin-4, retinoic acid, parathyroid hormone, MAPK inhibitors, such as, for

example, compounds disclosed in U.S. Published Application 2004/0209901 and U.S. Published Application 2004/0132729.

The pluripotent stem cells may be differentiated into an insulin-producing cell prior to transplantation into a recipient. In a specific embodiment, the pluripotent stem cells are fully differentiated into β-cells, prior to transplantation into a recipient. Alternatively, the pluripotent stem cells may be transplanted into a recipient in an undifferentiated or partially differentiated state. Further differentiation may take place in the recipient.

Definitive endoderm cells or, alternatively, pancreatic endoderm cells, or, alternatively, β cells, may be implanted as dispersed cells or formed into clusters that may be infused into the hepatic portal vein. Alternatively, cells may be provided in biocompatible degradable polymeric supports, porous non-degradable devices or encapsulated to protect from host immune response. Cells may be implanted into an appropriate site in a recipient. The implantation sites include, for example, the liver, natural pancreas, renal subcapsular space, omentum, peritoneum, subserosal space, intestine, stomach, or a subcutaneous pocket.

To enhance further differentiation, survival or activity of the implanted cells, additional factors, such as growth factors, antioxidants or anti-inflammatory agents, can be administered before, simultaneously with, or after the administration of the cells. In certain embodiments, growth factors are utilized to differentiate the administered cells in vivo. These factors can be secreted by endogenous cells and exposed to the administered cells in situ. Implanted cells can be induced to differentiate by any combination of endogenous and exogenously administered growth factors known in the art.

The amount of cells used in implantation depends on a number of various factors including the patient's condition and response to the therapy, and can be determined by one skilled in the art.

In one aspect, this invention provides a method for treating a patient suffering from, or at risk of developing diabetes. This method involves culturing pluripotent stem cells, differentiating the cultured cells in vitro into a β-cell lineage, and incorporating the cells into a three-dimensional support. The cells can be maintained in vitro on this support prior to implantation into the patient. Alternatively, the support containing the cells can be directly implanted in the patient without additional in vitro culturing. The support can optionally be incorporated with at least one pharmaceutical agent that facilitates the survival and function of the transplanted cells.

Support materials suitable for use for purposes of the present invention include tissue templates, conduits, barriers, and reservoirs useful for tissue repair. In particular, synthetic and natural materials in the form of foams, sponges, gels, hydrogels, textiles, and nonwoven structures, which have been used in vitro and in vivo to reconstruct or regenerate biological tissue, as well as to deliver chemotactic agents for inducing tissue growth, are suitable for use in practicing the methods of the present invention. See, for example, the materials disclosed in U.S. Pat. No. 5,770,417, U.S. Pat. No. 6,022,743, U.S. Pat. No. 5,567,612, U.S. Pat. No. 5,759,830, U.S. Pat. No. 6,626,950, U.S. Pat. No. 6,534,084, U.S. Pat. No. 6,306,424, U.S. Pat. No. 6,365,149, U.S. Pat. No. 6,599, 323, U.S. Pat. No. 6,656,488, U.S. Published Application 2004/0062753 A1, U.S. Pat. No. 4,557,264 and U.S. Pat. No. 6,333,029.

To form a support incorporated with a pharmaceutical agent, the pharmaceutical agent can be mixed with the polymer solution prior to forming the support. Alternatively, a pharmaceutical agent could be coated onto a fabricated sup-

port, preferably in the presence of a pharmaceutical carrier. The pharmaceutical agent may be present as a liquid, a finely divided solid, or any other appropriate physical form. Alternatively, excipients may be added to the support to alter the release rate of the pharmaceutical agent. In an alternate embodiment, the support is incorporated with at least one pharmaceutical compound that is an anti-inflammatory compound, such as, for example compounds disclosed in U.S. Pat. No. 6,509,369.

The support may be incorporated with at least one pharmaceutical compound that is an anti-apoptotic compound, such as, for example, compounds disclosed in U.S. Pat. No. 6,793,945.

The support may also be incorporated with at least one pharmaceutical compound that is an inhibitor of fibrosis, such as, for example, compounds disclosed in U.S. Pat. No. 6,331, 298.

The support may also be incorporated with at least one pharmaceutical compound that is capable of enhancing angiogenesis, such as, for example, compounds disclosed in U.S. Published Application 2004/0220393 and U.S. Published Application 2004/0209901.

The support may also be incorporated with at least one pharmaceutical compound that is an immunosuppressive compound, such as, for example, compounds disclosed in U.S. Published Application 2004/0171623.

The support may also be incorporated with at least one pharmaceutical compound that is a growth factor, such as, for example, members of the TGF-β family, including TGF-β1, 2, and 3, bone morphogenic proteins (BMP-2, -3, -4, -5, -6, -7, -11, -12, and -13), fibroblast growth factors-1 and -2, platelet-derived growth factor-AA, and -BB, platelet rich plasma, insulin growth factor (IGF-I, II) growth differentiation factor (GDF-5, -6, -8, -10, -15), vascular endothelial cell-derived growth factor (VEGF), pleiotrophin, endothelin, among others. Other pharmaceutical compounds can include, for example, nicotinamide, hypoxia inducible factor 1-alpha, glucagon like peptide-I (GLP-1), GLP-1 and GLP-2 mimetibody, and II, Exendin-4, nodal, noggin, NGF, retinoic acid, parathyroid hormone, tenascin-C, tropoelastin, thrombin-derived peptides, cathelicidins, defensins, laminin, biological peptides containing cell- and heparin-binding domains of adhesive extracellular matrix proteins such as fibronectin and vitronectin, MAPK inhibitors, such as, for example, compounds disclosed in U.S. Published Application 2004/0209901 and U.S. Published Application 2004/0132729.

The incorporation of the cells of the present invention into a scaffold can be achieved by the simple depositing of cells onto the scaffold. Cells can enter into the scaffold by simple diffusion (J. Pediatr. Surg. 23 (1 Pt 2): 3-9 (1988)). Several other approaches have been developed to enhance the efficiency of cell seeding. For example, spinner flasks have been used in seeding of chondrocytes onto polyglycolic acid scaffolds (Biotechnol. Prog. 14(2): 193-202 (1998)). Another approach for seeding cells is the use of centrifugation, which yields minimum stress to the seeded cells and enhances seeding efficiency. For example, Yang et al. developed a cell seeding method (J. Biomed. Mater. Res. 55(3): 379-86 (2001)), referred to as Centrifugational Cell Immobilization (CCI).

The present invention is further illustrated, but not limited by, the following examples.

EXAMPLES

Example 1

Formation of a Population of Cells Expressing Markers Characteristic of the Pancreatic Endocrine Lineage that Co-Express Insulin and NKX6.1 and Minimal Amounts of Glucagon

Cells of the human embryonic stem cells line H1 were cultured on MATRIGEL® (1:30 dilution) (BD Biosciences; Cat #356231)-coated dishes with RPMI medium (Invitrogen; Cat #: 22400)+0.2% FBS+100 ng/ml activin A (PeproTech; Cat #120-14)+20 ng/ml WNT-3a (R&D Systems; Cat #1324-WN/CF) for one day followed by treatment with RPMI media+0.5% FBS+100 ng/ml activin A for an additional two days (Stage 1), then,

   a. DMEM/F12 (Invitrogen; Cat #11330-032)+2% FBS+50 ng/ml FGF7 (PeproTech; Cat #100-19) for three days (Stage 2), then
   b. DMEM-High glucose (Invitrogen; Cat #10569)+1% B27+50 ng/ml FGF7+0.25 µM Cyclopamine-KAAD (Calbiochem; Cat #239804)+100 ng/ml Noggin (R&D Systems; Cat #3344-NG) for four days (Stage 3), then
   c. DMEM-High glucose+1% B27 (Invitrogen; Cat #0791)+100 ng/ml Noggin+1 µM ALK5 inhibitor II (Axxora; Cat # ALX-270-445)+500 nM TBP ((2S,5S)-(E,E)-8-(5-(4-(Trifluoromethyl)phenyl)-2,4-pentadi-enoylamino)benzolactam) (Calbiochem; Cat #565740) for six days (Stage 4).

As a control, separate populations of cells were treated with DMEM-High glucose+1% B27+100 ng/ml Noggin+1 µM ALK5 inhibitor II for six days (Stage 4, control group).

As shown in FIG. 1, TBP treatment at stage 4 resulted in an increase of insulin-expressing cells (FIG. 1 panel a). It was noted that about 60% of these insulin expressing cells are single endocrine hormone expressing cells, wherein the cells expressed insulin and did not express glucagon somatostatin and ghrelin (FIG. 1 panel a and b, FIG. 5 panel d and e). Glucagon-expressing cells were also noted in the cultures that received TBP treatment. Most of the glucagon-expressing cells also co-expressed insulin (FIG. 1 panel a and b). For the control group, the majority of the cells co-expressed insulin and glucagon (FIG. 1 panel c and d).

In a separate experiment, cells of the human embryonic stem cells line H1 were cultured on MATRIGEL® (1:30 dilution) coated dishes with RPMI medium+0.2% FBS+100 ng/ml activin A+20 ng/ml WNT-3a for one day followed by treatment with RPMI media+0.5% FBS+100 ng/ml activin A for an additional two days (Stage 1), then

   a. DMEM/F12+2% FBS+50 ng/ml FGF7 for three days (Stage 2), then
   b. DMEM-High glucose+1% B27+0.25 µM Cyclopam-ine–KAAD+2 µM Retinoic acid (RA)+100 ng/ml of Noggin for four days (Stage 3), then
   c. Treatment 1: DMEM-High glucose+1% B27+100 ng/ml Noggin+1 µM ALK5 inhibitor II+500 nM TBP for six days (Stage 4, Treatment 1), or
   d. Treatment 2: DMEM-High glucose+1% B27+100 ng/ml Noggin+1 µM ALK5 inhibitor II+100 nM TBP for six days (Stage 4, Treatment 2), or
   e. Treatment 3: DMEM-High glucose+1% B27+100 ng/ml Noggin+1 µM ALK5 inhibitor II+20 nM TBP for six days (Stage 4, Treatment 3), or
   f. Treatment 4: DMEM-High glucose+1% B27+100 ng/ml Noggin+1 µM ALK5 inhibitor II for six days (Stage 4, Treatment 4).

Immunocytochemistry analysis was used to assess the effects of the different concentrations of TPB on the formation of the cells of the present invention. Significant increases in the number of single insulin-expressing cells was observed in both 500 nM and 100 nM TPB treatment groups (FIG. 2 panel a and b). FACS analysis confirmed that both treatments gave rise to 12% single insulin expressing cells in vitro and 15% of that population also expressed NKX6.1 (Table 1—NKX6.1/INS expressing cells were 2.4% of the total population). At 20 nM TPB, similar to the control group, most cells co-expressed insulin and glucagon (FIG. 2 panel c and d).

TABLE 1

Expression of markers characteristic of the pancreatic endocrine lineage, shown as a percentage of the total cell population.

	Synaptophysin	INS	NKX6.1	NKX6.1/INS
TPB (500 nM)	38.3%	9.4%	45.7%	2.4%
TPB (100 nM)	47.6%	14.4%	34.8%	3.1%

In order to further confirm that the effect on endocrine hormone expressing cell formation was mediated by the activation of protein kinase C, separate populations of cells of the human embryonic stem cells line H1 were cultured on MATRIGEL® (1:30 dilution) coated dishes with RPMI medium+0.2% FBS+100 ng/ml activin A+20 ng/ml WNT-3a for one day followed by treatment with RPMI media+0.5% FBS+100 ng/ml activin A for an additional two days (Stage 1), then

   a. DMEM/F12+2% FBS+50 ng/ml FGF7 for three days (Stage 2), then
   b. DMEM-High glucose+1% B27+0.25 µM Cyclopam-ine–KAAD+2 µM Retinoic acid (RA)+100 ng/ml of Noggin for four days (Stage 3), then
   c. Treatment 5: DMEM-High glucose+1% B27+100 ng/ml Noggin+1 µM ALK5 inhibitor II+500 nM TBP for six days (Stage 4, Treatment 5), or
   d. Treatment 6: DMEM-High glucose+1% B27+100 ng/ml Noggin+1 µM ALK5 inhibitor II+500 nM TPB+5 µM GÖ 6976 for six days (Stage 4, Treatment 6), or
   e. Treatment 7: DMEM-High glucose+1% B27+100 ng/ml Noggin+1 µM ALK5 inhibitor II (Stage 4, Treatment 7), then
   f. DMEM-High glucose+1% B27 for four days (Stage 5).

GÖ 6976 is known to selectively inhibit Ca^{2+}-dependent protein kinase C isoforms. A significant decrease in the number cells expressing markers characteristic of the pancreatic endocrine lineage, in cultures receiving TPB alone (FIG. 3, panel a) and TPB and GÖ 6976 (FIG. 3, panel b). FACS analysis confirmed that TPB treatment (Treatment 6) gave rise to 30.6% synaptophysin, 12% single insulin and 4.6% glucagon expressing cells. On the other hand, TBP and GÖ 6976 treatment (Treatment 7) gave rise to 10.6% synaptophysin and no detectable level of single insulin expressing cells (Table 2). There was no difference in the total number of cells observed between Treatment 6 and Treatment 7. (See FIG. 3, panels c and d, showing DAPI staining, reflecting total cell number in Treatment 6 and Treatment 7). These results suggest that protein kinase C signaling may be important for the formation of cells expressing markers characteristic of the pancreatic endocrine lineage.

Other protein kinase C activators were also tested. These were Indolactam V (ILV) (Axxora; Cat #ALX-420-011-

C300) and phorbol-12-myristate-13-acetate (PMA) (Calbiochem; Cat#524400). However, only TPB demonstrated the formation of single insulin expressing cells (FIG. **4**, panel a). Both ILV (FIG. **4**, panel b) and PMA (FIG. **4**, panel c) at 500 nM, gave rise to cells co-expressing insulin and glucagon after six days. FACS analysis confirmed that TPB treatment gave rise to 12% single insulin expressing, and 4.6% glucagon expressing cells and 7.1% insulin and glucagon co-expressing cells. On the other hand, ILV treatment gave rise to 3% single insulin expressing, and 12% glucagon cells and 12% insulin and glucagon co-expressing cells (Table 2) Immunocytochemistry analysis showed that in cultures treated with TPB, 20% of the insulin expressing cells co-expressed NKX6.1 (FIG. **5** panel a) and PDX1 (FIG. **5** panel b). The majority of the insulin expressing cells co-expressed NEUROD, an endocrine maker (FIG. **5** panel c). Very few of the insulin expressing cells co-expressed somatostatin or ghrelin (GHRL) (FIG. **5** panel d and e).

TABLE 2

Expression of markers characteristic of the pancreatic endocrine lineage, shown as a percentage of the total cell population.

	TPB	ILV	TPB + Go6976
Synaptophysin	30.6%	56.8%	10.6%
INS	12%	3%	—
GCG	4.6%	12.6%	3.1%
INS/GCG	7.1%	12.9%	4%

Example 2

An Alternative Method for the Formation of a Population of Cells Expressing Markers Characteristic of the Pancreatic Endocrine Lineage that Co-Express Insulin and NKX6.1 and Minimal Amounts of Glucagon

In a separate experiment, cells of the human embryonic stem cells line H1 were cultured on MATRIGEL® (1:30 dilution) coated dishes with RPMI medium+0.2% FBS+100 ng/ml activin A+20 ng/ml WNT-3a for one day followed by treatment with RPMI media+0.5% FBS+100 ng/ml activin A for an additional two days (Stage 1), then
   a. DMEM/F12+2% FBS+50 ng/ml FGF7 for three days (Stage 2), then
   b. Treatment 8: DMEM-High glucose+1% B27+50 ng/ml FGF7+0.25 µM Cyclopamine–KAAD+2 µM Retinoic acid (RA)+100 ng/ml of Noggin+20 ng/ml Activin A+a p38 kinase inhibitor (disclosed in U.S. Pat. No. 6,214, 830, at 2.5 µM) for four days (Stage 3, Treatment 8), or
   c. Treatment 9: DMEM-High glucose+1% B27+0.25 µM Cyclopamine-KAAD+2 µM Retinoic acid (RA)+100 ng/ml of Noggin for four days (Stage 3, Treatment 9), then
   d. DMEM-High glucose+1% B27+100 ng/ml Noggin+1 µM ALK5 inhibitor II+500 nM TPB for six days (Stage 4).

Stage 3, Treatment 8 resulted in the formation of a population of cells expressing markers characteristic of the pancreatic endoderm lineage that co-expressed PDX1 and NKX6.1, but did not express CDX2 and NGN3. On the other hand, stage 3, treatment 9 resulted in the formation of a population of cells expressing markers characteristic of the pancreatic endoderm lineage that co-expressed PDX1,

NKX6.1 and NGN3. The effects of treatment with protein kinase C activator treatment on these cell populations were examined (Stage 4 above).

FACS analysis was performed to ascertain the percentage of insulin single positive cells, glucagon single positive cells, insulin/glucagon double positive cells, cells expressing NKX6.1 positive cells, insulin/NKX6.1 positive cells, and synaptophysin positive cells (a pan endocrine marker).

As shown in Table 3, the cell population formed with Treatment 8 gave rise to a larger percentage of endocrine cells, as denoted by synaptophysin expression: 49.7% of the total cell population expressed synaptophysin. 27.8% of the total population was insulin single positive cells.

On the other hand, the cell population formed with treatment 9 only gave rise to 25.7% synaptophysin expressing cells. 7.6% of the total population was single insulin-expressing cells. No significant difference of single glucagon expressing cells was observed in both treatments and the percentage of glucagon expressing cells was significantly lower than the insulin expressing cells.

A significant amount of insulin-expressing cells also co-expressed NKX6.1. In populations of cells that received treatment 8, 11% of the total population expressed insulin and NKX6.1. In populations of cells that received treatment 9, 2% of the total population expressed insulin and NKX6.1.

Immunofluorescent analysis confirmed the above (FIG. **6**). Treatment 8, resulted in an increase of insulin expressing cells comparing to Treatment 9 (FIG. **6** panel a and d). Most glucagon expressing cells were poly-hormonal cells (FIG. **6**, panel a, b, d and e). These results suggest that the population of cells generated by treatment 8 (cells expressing makers characteristic of the pancreatic endoderm lineage that co-expressed PDX1 and NKX6.1, but did not express CDX2 and NGN3) can be more efficiently induced to become mature and functional insulin expressing cells by the methods of the present invention.

TABLE 3

Expression of markers characteristic of the pancreatic endocrine lineage, shown as a percentage of the total cell population.

	Synaptophysin	Insulin	Glucagon	Insulin/ Glucagon	NKX6.1	NKX6.1/ Insulin
T8	49.7%	27.8%	2.0%	16.4%	44.2%	11.0%
T9	25.7%	7.6%	2.5%	4.9%	61.7%	2.0%

Example 3

An Alternative Method for the Formation of a Population of Cells Expressing Markers Characteristic of the Pancreatic Endocrine Lineage that Co-Express Insulin and NKX6.1 and Minimal Amounts of Glucagon

In another experiment, cells of the human embryonic stem cells line H1 were cultured on MATRIGEL® (1:30 dilution) coated dishes with RPMI medium+0.2% FBS+100 ng/ml activin A+20 ng/ml WNT-3a for one day followed by treatment with RPMI media+0.5% FBS+100 ng/ml activin A for an additional two days (Stage 1), then
   a. DMEM/F12+2% FBS+50 ng/ml FGF7 for three days (Stage 2), then
   b. DMEM-High glucose+1% B27+50 ng/ml FGF7+0.25 µM Cyclopamine–KAAD+2 µM Retinoic acid (RA)+

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100 ng/ml of Noggin+20 ng/ml Activin A+a p38 kinase inhibitor (JNJ3026582, at 2.5 μM) for four days (Stage 3), then

c. Treatment 10: DMEM-High glucose+1% B27+100 ng/ml Noggin+1 μM ALK5 inhibitor II+500 nM TPB for six days (Stage 4, Treatment 10), or

d. Treatment 11: DMEM-High glucose+1% B27+100 ng/ml Noggin+1 μM ALK5 inhibitor II+500 nM TPB for nine days (Stage 4, Treatment 11), or

e. Treatment 12: DMEM-High glucose+1% B27+100 ng/ml Noggin+1 μM ALK5 inhibitor II+500 nM TPB for twelve days (Stage 4, Treatment 12)

As shown in Table 4, when the duration of the protein kinase C activator treatment was extended to either nine days (Treatment 11) or twelve days (Treatment 12), no additional benefit was observed. Single insulin-expressing cells were 27.8% of the total population after six days treatment with Treatment 10. Conversely, insulin-expressing cells decreased to 10% after nine days treatment (Treatment 11), and declined further to 4% after twelve days treatment (Treatment 12). In parallel, the total percentage of insulin and NKX6.1 co-expressing cell population also dropped significantly after extending the treatment. These results suggested that a six day treatment with Noggin, Alk5 inhibitor II and a protein kinase C activator was sufficient enough to form the cells of the present invention.

TABLE 4

Expression of markers characteristic of the pancreatic endocrine lineage, shown as a percentage of the total cell population.

	Synaptophysin	Insulin	Glu-cagon	Insulin/Glu-cagon	NKX6.1	NKX6.1/Insulin
6-day	49.7%	27.8%	2.0%	16.4%	44.2%	11.0%
9-day	43.5%	10.0%	6.6%	7.8%	33.5%	1.0%
12-day	37.6%	4.4%	4%	6.3%	32.5%	1.0%

Example 4

Implantation of the Cells of the Present Invention into Severe Combined Immunodeficient (SCID)—Beige (Bg) Mice

Cells of the human embryonic stem cells line H1 were cultured on MATRIGEL® (1:30 dilution)—coated dishes with RPMI medium+0.2% FBS+100 ng/ml activin A+20 ng/ml WNT-3a for one day followed by treatment with RPMI media+0.5% FBS+100 ng/ml activin A for an additional two days (Stage 1), then,

a. DMEM/F12+2% FBS+50 ng/ml FGF7 for three days (Stage 2), then

b. DMEM-High glucose+1% B27+50 ng/ml FGF7+0.25 μM Cyclopamine–KAAD+100 ng/ml Noggin for four days (Stage 3), then

c. DMEM-High glucose+1% B27+100 ng/ml Noggin+1 μM ALK5 inhibitor II+500 nM TBP for six days (Stage 4).

Cells at the end of stage four were mechanically scored using a 1-ml glass pipette and subsequently transferred to non-adherent plates for culture overnight. The resulting cell aggregates were collected, and aggregates containing 5 million cells were transplanted into the kidney capsule of an immuno-compromised mice (SCID/Bg, animal Nos. 47. 48. 49, 50 and 51). See FIG. 7.

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After four weeks, functionality of the insulin-producing cells in these grafts was tested by injecting animals with glucose to induce insulin secretion. The animals were fasted for about 15-20 hrs, after which a blood sample (pre-glucose) was withdrawn retro-orbitally. Each animal then received an intraperitoneal injection dose of approximately 3 g/kg of glucose in 30% dextrose solution, and blood was withdrawn at about 60 minutes post glucose infusion. Circulating human C-peptide was detected using in mouse serum using an ultra-sensitive human specific C-peptide ELISA plates (Cat No. 80-CPTHU-E01, Alpco Diagnostics, NH). The detection of human C-peptide indicates that insulin secretion is derived from the grafted cells.

Human C-peptide was detected in animal serum as early as 4 weeks after transplantation and increased over time. The transplantation data is summarized in FIG. 7. At the end of one month, we were able to detect the human C-peptide (less than 0.2 ng/ml) in response to glucose administration in 60% of the animals in the study group. Glucose stimulated serum level of human C-peptide increased 5 to 10 fold in three out of the four mice after four weeks. At twelve weeks post implantation, the average glucose-stimulated serum levels of human c-peptide in transplanted mice were greater than 1 mg/ml (n=4).

Example 5

An Alternative Method for the Formation of a Population of Cells Expressing Markers Characteristic of the Pancreatic Endoderm Lineage that Co-Express PDX1 and NKX6.1

Briefly, cells of the human embryonic stem cell line H1 were cultured on MATRIGEL® (1:30 dilution) coated dishes and RPMI medium supplemented with 0.2% FBS, 100 ng/ml activin A and 20 ng/ml WNT-3a for one day, followed by treatment with RPMI media supplemented with 0.5% FBS and 100 ng/ml activin A, for an additional two days (Stage 1), then

a. DMEM/F12+2% FBS+50 ng/ml FGF7 for three days (Stage 2), then

b. DMEM-High glucose+1% B27+0.25 μM Cyclopamine–KAAD+2 μM Retinoic acid (RA)+100 ng/ml of Noggin for four days (Stage 3), then

c. DMEM-High glucose+1% B27+100 ng/ml Noggin+1 μM ALK5 inhibitor II+20 nM PMA, or 100 nM TPB, or 20 nM Phorbol-12,13-dibutyrate (PDBu) (Calbiochem, cat#524390) for six days (Stage 4)

As a control, separate populations of cells were treated with DMEM High glucose, supplemented with 1% B27, 100 ng/ml of Noggin and 1 μM ALK5 inhibitor II for six days (stage 4).

Cultures were sampled in duplicate on stage 4 day 6, and imaging was performed using an IN Cell Analyzer 1000 (GE Healthcare). Images from 100 fields per well were acquired to compensate for any cell loss during the bioassay and subsequent staining procedures. Measurements for total cell number, total PDX1 expressing cells and total NKX6.1 expressing cell were obtained from each well using IN Cell Developer Toolbox 1.7 (GE Healthcare) software. Averages and standard deviations were calculated for each replicate data set. Total PDX1 and NKX6.1 protein expressing cells was reported as percentage of the total cell population. As shown in FIG. 8, there was a dramatic increase of NKX6.1/PDX1 expressing cell population in the protein kinase C activator treated groups at a lower effective concentration (approximately 20 nM), compared to samples obtained from the con-

trol treatment. By day 6 of Stage 4, in populations of cells that received either protein kinase C activator or control treatment, 92%±4% of the population expressed PDX1. In the protein kinase C activator treated group, 75%±5% PDX1-expressing cells expressed NKX6.1. However, in populations only treated with Noggin and TGF beta receptor inhibitor (control), only 58%±5% of the PDX1-expressing cells expressed NKX6.1. In the presence of protein kinase C activator, 20% NKX6.1-expressing cells were co-positive with proliferation marker, EdU (Click-iT® EdU Imaging Kit, Invitrogen, Cat#C10337).

This example demonstrates that a protein kinase C activator can be used in combination with Noggin and TGF beta receptor inhibitor at a relatively low effective concentration (~20 nM) to facilitate the up-regulation of Nkx6.1 expression, and increase the percentage of cells expressing PDX1 and NKX6.1.

Example 6

Treatment of Cells Expressing Markers Characteristic of the Pancreatic Endoderm Lineage with Protein Kinase C Activators

Briefly, cells of the human embryonic stem cell line H1 were cultured on MATRIGEL® (1:30 dilution) coated dishes and RPMI medium supplemented with 0.2% FBS, 100 ng/ml activin A and 20 ng/ml WNT-3a for one day, followed by treatment with RPMI media supplemented with 0.5% FBS and 100 ng/ml activin A, for an additional two days (Stage 1), then

    a. DMEM/F12+2% FBS+50 ng/ml FGF7 for three days (Stage 2), then

    b. T1: DMEM-High glucose+1% B27+0.25 μM Cyclopamine–KAAD+2 μM Retinoic acid (RA)+100 ng/ml of Noggin+FGF10 50 ng/ml for four days (Stage 3, T1) or,

      T2: DMEM-High glucose+1% B27+0.25 μM Cyclopamine–KAAD+2 μM Retinoic acid (RA)+100 ng/ml of Noggin+FGF10 50 ng/ml+100 nM TPB for four days (Stage 3, T2), then

    c. DMEM-High glucose+1% B27+100 ng/ml Noggin+1 μM ALK5 inhibitor II for six days (Stage 4)

As shown in FIG. **9**, a significant down-regulation of the pancreatic endoderm markers PDX1, NKX6.1 and PTF1 alpha was observed, in cells treated with TPB (T2) compared to the control group (T1). NKX6.1 was undetectable by immunohistochemistry. These data suggest that protein kinase activator treatment at stage 3 did not facilitate the generation of PDX1/NKX6.1 co-expressing cells.

Publications cited throughout this document are hereby incorporated by reference in their entirety. Although the various aspects of the invention have been illustrated above by reference to examples and preferred embodiments, it will be appreciated that the scope of the invention is defined not by the foregoing description but by the following claims properly construed under principles of patent law.

What is claimed is:

1. A method to generate a population of pancreatic endocrine cells that co-express NKX6.1 and insulin, the method comprising the steps of:

    a) culturing human pluripotent stem cells;

    b) differentiating the human pluripotent stem cells of step a) into definitive endoderm cells by culturing the human pluripotent stem cells in medium comprising a TGF-β receptor agonist;

    c) differentiating the definitive endoderm cells of step b) into pancreatic endoderm cells by culturing the definitive endoderm cells in the presence of an FGF family member and retinoic acid; and

    d) differentiating the pancreatic endoderm cells of step c) into pancreatic endocrine cells by culturing the pancreatic endoderm cells in a medium supplemented with noggin, an inhibitor of ALK5 and a protein kinase C activator, wherein the pancreatic endocrine cells co-express NKX6.1 and insulin and wherein less than 10% of the cells in the population express glucagon.

2. The method of claim **1**, wherein the inhibitor of ALK5 in step d) is ALK5 inhibitor II.

3. The method of claim **1**, wherein the protein kinase C activator in step d) is (2S,5S)-(E,E)-8-(5-(4-(Trifluoromethyl)phenyl)-2,4-pentadienoylamino)benzolactam.

4. The method of claim **1**, wherein at least 30% of the cells express NKX6.1.

5. The method of claim **1**, wherein at least 40% of the cells express NKX6.1.

6. The method of claim **1**, wherein at least 50% of the cells express NKX6.1.

7. The method of claim **1**, wherein at least 60% of the cells express NKX6.1.

8. The method of claim **1**, wherein at least 5% of the cells express insulin.

9. A method to generate a population of pancreatic endocrine cells that co-express NKX6.1 and insulin, the method comprising the steps of:

    a) culturing human pluripotent stem cells;

    b) differentiating the human pluripotent stem cells of step a) into definitive endoderm cells by culturing the human pluripotent stem cells in medium comprising a TGF-β receptor agonist;

    c) differentiating the definitive endoderm cells of step b) into pancreatic endoderm cells by culturing the definitive endoderm cells in the presence of an FGF family member and retinoic acid; and

    d) differentiating the pancreatic endoderm cells of step c) into pancreatic endocrine cells by culturing the pancreatic endoderm cells in a medium supplemented with noggin, a TGF-β receptor signaling inhibitor and (2S, 5S)-(E,E)-8-(5-(4-(Trifluoromethyl)phenyl)-2,4-pentadienoylamino)benzolactam, wherein the pancreatic endocrine cells co-express NKX6.1 and insulin and wherein less than 10% of the cells in the population express glucagon.

10. The method of claim **9**, wherein at least 30% of the cells express NKX6.1.

11. The method of claim **9**, wherein at least 40% of the cells express NKX6.1.

12. The method of claim **9**, wherein at least 50% of the cells express NKX6.1.

13. The method of claim **9**, wherein at least 60% of the cells express NKX6.1.

14. The method of claim **9**, wherein at least 5% of the cells express insulin.

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